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Enzymatic Breakdown of Type II Collagen in the Human Vitreous

Mariëlle van Deemter,¹ Hendri H. Pas,² Roel Kuijer,³ Roelofje J. van der Worp,^{1,3} Johanna M. M. Hooymans,¹ and Leonoor I. Los¹

PURPOSE. To investigate whether enzymatic collagen breakdown is an active process in the human vitreous.

METHODS. Human donor eyes were used for immunohistochemistry to detect the possible presence of the matrix metalloproteinase (MMP)-induced type II collagen breakdown product col2-3/4C-short in the vitreous. Western blot and slot blot analyses were used to further identify vitreal type II collagen breakdown products in three age groups with average ages of 25, 45, and 65 years. Purified type II collagen was cleaved by MMPs that are known to occur naturally in the vitreous to elucidate what possible type II collagen breakdown products could thus be formed in the human vitreous.

RESULTS. By means of both immunohistochemistry and slot blot analysis, col2-3/4C-short was detected in the vitreous. Using Western blot analysis, a range of type II collagen breakdown products was found, mostly in younger eyes, but none of these products contained the neopeptide that characterizes the col2-3/4C-short molecule. Digestion of purified type II collagen by MMPs did not give the same breakdown products as found in the vitreous.

CONCLUSIONS. The presence of collagen degradation products in the human vitreous supports the hypothesis that enzymatic breakdown is most likely an active process in this extracellular matrix. Based on the size of the degradation products found by Western blot analysis, it is likely that in addition to MMPs, other proteolytic enzymes able to digest type II collagen are also active. (*Invest Ophthalmol Vis Sci.* 2009;50:4552–4560) DOI: 10.1167/iovs.08-3125

The vitreous body is the gel-like extracellular matrix in the eye that fills the space between the lens and the retina. It has functions in eye growth, in applying turgor pressure, and in maintaining the eye's optical transparency by preventing cells from entering the vitreous cavity. With a water content of 98% to 99%, it is highly hydrated. Furthermore, the low protein concentration minimizes light scattering, thus contributing to optimized vision.^{1,2} With aging, the vitreous body

undergoes structural changes, resulting in the formation of liquid-filled spaces (synchysis) and optically dense condensations within the matrix (syneresis).^{3,4} Liquefaction and collapse of the matrix can be followed by posterior vitreous detachment^{5–8} that can induce pathologic conditions such as retinal tears, retinal detachment, and intravitreal hemorrhage.⁹

The most important structural components of the vitreous body are collagens. These are assembled as fibrils, consisting of a type V/XI collagen core surrounded by type II collagen and a coating of type IX collagen.^{10,11} Type II collagen is predominant and consists of approximately 80% of total vitreous collagen. In vitro studies in ox vitreous¹² and in vivo studies in chick vitreous¹³ have shown that when vitreous collagen is broken down enzymatically the gel structure disappears. Hydration of the gel is maintained primarily by the presence of hyaluronan, a high molecular weight glycosaminoglycan capable of binding large quantities of water.¹⁴ Proteoglycans, of which type IX collagen is one, apparently maintain the spacing between collagen fibrils.³

Previous studies speculated that the mechanisms underlying synchysis and syneresis were interrelated. Initially it was speculated that morphologic changes of the vitreous are caused by the mechanical unmixing of the collagenous and non-collagenous components.¹ Later, Bishop^{3,10} hypothesized that synchysis and syneresis may be caused by the gradual loss of proteoglycan coating from collagen fibrils that is seen with aging. Sticky collagen fibrils would then be able to contact each other and aggregate; simultaneously, the areas that consequently lacked the presence of collagen fibrils would be converted from a gel to a liquid phase. However, in a light and electron microscopic study by Los et al.⁴ on structural changes in the vitreous, no prominent aggregation of intact collagen fibrils was observed in the vicinity of the liquefied spaces. Instead, fragmentation of the collagen fibrils was detected. Bishop³ hypothesized that enzymes were likely to be involved in the loss of proteoglycans from the collagen fibrils. In line with this, we propose that enzymatic degradation may also be involved in the fragmentation of type II collagen in the vitreous.

Given that the vitreous is an extracellular matrix (ECM), it seems plausible that the fragmentation of the collagen fibrils is caused by endogenous breakdown induced by matrix-related proteolytic enzymes. Important enzymes involved in the remodeling and breakdown of ECMs are the matrix metalloproteinases (MMPs), which together can break down all ECM components. In cartilage, MMP-13 is the most likely enzyme to degrade type II collagen because it is produced by chondrocytes and cleaves type II collagen far more efficiently than the other collagenases.^{15,16} The first cleavage occurs at gly⁷⁷⁵-leu/ile⁷⁷⁶, thereby generating the characteristic 3/4 N-terminal and 1/4 C-terminal fragments.¹⁷ MMP-1 and MMP-8 are also capable of cleaving intact type II collagen at the same site. After this initial cleavage, the triple helical structure is lost, and the collagen becomes susceptible to further enzymatic breakdown by other enzymes. Several MMPs have been discovered in the vitreous, among them the collagenases MMP-1 and MMP-8 and the gelatinases MMP-2 and MMP-9.^{18,19}

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Our study focuses on whether enzymatic collagen breakdown is an active process in the human vitreous. Therefore, in several age groups we analyzed whether, in addition to intact collagen triple helices, collagen breakdown products also occur naturally in the vitreous body. We demonstrate that fragments of collagen are indeed present. In addition we analyzed the possible involvement of MMPs in vitreous collagen fragmentation.

MATERIALS AND METHODS

Human eyes with no known ophthalmic disorders were obtained from the Cornea Bank (Amsterdam, The Netherlands) after removal of the corneas for transplantation ($n = 39$; age range, 15–74 years). Information on refractive errors of the eyes or their axial lengths before removal of the corneas was not available. However, eyes that seemed to be exceptionally large or small were not included in this study. Three specimens were used for histology (ages 55, 69, and 74 years) and thus were embedded in paraffin within 48 hours of death; all others were used for analysis of type II collagen breakdown products. Isolation of the vitreous bodies was performed by first cleaving the sclera. Then the choroid, lens, and retina were removed. Finally, the pars plana remnants of the ciliary body were carefully cleaved. The isolated vitreous bodies were washed and frozen at -80°C , within 48 hours of death. Before analysis, pools were formed of four vitreous bodies each, arranged according to age group. Three age groups were formed: P_{25} , average age 25 years; P_{45} , average age 45 years; and P_{65} , average age 65 years.

Porcine eyes ($n = 6$), obtained from a local abattoir, were used for analysis of the effect of postmortem time on type II collagen and its breakdown products. Vitreous bodies were isolated within 2 hours of death or after 24 hours or 1 week of storage at 4°C ($n = 2$ of each time point). Isolation and analysis of type II collagen breakdown products was then performed.

Human cartilage obtained from the knees of two patients with osteoarthritis was used for the preparation of positive and negative controls for immunohistochemistry and Western blot analysis. Areas of healthy and degenerated cartilage in one sample were identified by macroscopic examination; regions or cartilage that looked macroscopically normal were defined as healthy cartilage, whereas soft, dull cartilage with lacerations was classified as osteoarthritic. Pieces of both were embedded in paraffin and used for immunohistochemical analysis. Nonclassified pieces of cartilage from the second sample were frozen and pulverized and used for collagen isolation. Unless otherwise mentioned, all reagents used were of analytical grade.

Paraffin Embedding and Immunohistochemistry

Human eyes and pieces of healthy knee cartilage and osteoarthritic, degenerated knee cartilage were embedded in paraffin. The samples were fixed overnight in 2% paraformaldehyde in phosphate-buffered saline (PBS) and then washed. This was followed by four 45-minute dehydration steps in 50% ethanol that were gradually increased to 100% ethanol and then by two additional 45-minute incubations in 100% ethanol. Next, the samples were incubated for 3×45 minutes in xylol, followed by a 1-hour incubation in paraffin at 60°C . Then the samples were incubated overnight in fresh paraffin at 60°C followed by embedding at room temperature.

Sagittal sections $5\text{-}\mu\text{m}$ thick were mounted on slides, dried overnight at 37°C , and underwent the following procedures. First, the sections were deparaffinized by incubation in xylol, 100% ethanol, 96% ethanol, and 70% ethanol and were washed in distilled water. Then the sections were treated for 30 minutes at room temperature with 0.01% type XXIV protease (Sigma-Aldrich, St. Louis, MO) and were washed in PBS. Sections were blocked by means of 30-minute incubation in PBS, 2% bovine serum albumin (BSA), and 5% serum (host of the secondary antibody) and were washed in PBS. The primary antibody was either mouse-anti-type II collagen Ab-3, which binds on the N-terminal 3/4

part of type II collagen (Lab Vision, Fremont, CA), or rabbit-anti-col2-3/4C-short (C12C Ab 2699; IBEX Diagnostics, Montreal, Canada), which specifically binds the neopeptide on the 3/4-end of the type II collagen fragment formed on cleavage with MMP-1, MMP-8, or MMP-13.¹⁷ Sections were incubated with antibodies diluted 1:50 in PBS, 1% BSA, for 1 hour at 37°C , followed by 3×5 -minute washes in PBS. Negative control sections were incubated in PBS and 1% BSA without the primary antibody. Then the sections were incubated in PBS and 0.1% H_2O_2 for 15 minutes, followed by washing in PBS. The secondary antibody was either horseradish peroxidase-conjugated rabbit-anti-mouse or horseradish peroxidase-conjugated swine-anti-rabbit (Dako, Carpinteria, CA), depending on the first antibody. Sections were incubated with antibodies diluted 1:100 in PBS, 1% BSA, 2% serum for 45 minutes at room temperature, and 3×5 -minute washes in PBS. Staining was then performed by incubating in 3-amino-9-ethylcarbazole (Sigma-Aldrich). The sections were briefly rinsed with distilled water, followed by staining with hematoxylin. After thorough rinsing with running tap water, Kaiser glycerol gelatin (Merck, Darmstadt, Germany) was added, and the sections were covered with a coverslip.

Isolation of Collagen and Collagen Fragments from Vitreous

Collagen was isolated according to a method adapted from Brown et al.²⁰ Pools consisting of four vitreous bodies each and subdivided into three different age groups were homogenized with a homogenizer (DIAX 600; Heidolph, Kelheim, Germany). This was followed by 2-hour centrifugation at 30,000g. The supernatants were stored at -80°C to determine the presence of type II collagen fragments, as described. The pellets were resuspended in 2 mL of 0.5 M acetic acid (HAc) containing 0.1% pepsin (Sigma-Aldrich) and incubated for 24 hours at 4°C while gently rotating. Insoluble proteins were removed from the solution by centrifuging for 30 minutes at 16,000g. Soluble proteins were obtained by adding crystalline NaCl in a final concentration of 3 M to the solution. After overnight incubation at 4°C under gentle rotation, the precipitates were centrifuged for 30 minutes at 16,000g and were dissolved in 0.5 M HAc. This was dialyzed against 0.1 M HAc, after which the protein concentration was determined by measuring the absorption at 280 nm (A_{280}) using a spectrophotometer (Nanodrop; Isogen, Maarssen, The Netherlands). The samples were lyophilized and stored at -80°C until further use. For all experiments the lyophilized samples were redissolved in 0.5 M HAc at a concentration of 1 mg/mL. These pepsin extracts of the different age groups were labeled P_{25-P} , P_{45-P} , and P_{65-P} .

Supernatants obtained at the first centrifugation step were used without further treatment (labeled P_{25-S} , P_{45-S} , and P_{65-S}) or, depending on the experiment, were concentrated by ultrafiltration (labeled P_{25-SC} , P_{45-SC} , and P_{65-SC}). Before concentrating, the supernatant was first filtered through a 50-kDa filter (Amicon Ultra; Millipore, Billerica, MA) so that larger proteins that could clot the 10-kDa filter (Amicon Ultra; Millipore) in the next step could be removed. The flow through of the 50-kDa filter was then concentrated 100 times with the 10-kDa filter.

Isolation of type II collagen and type II collagen breakdown products from the separate porcine vitreous bodies was performed in a way similar to the method described, except that the pellets were resuspended in 0.5 mL 0.1% pepsin in 0.5 M HAc. Porcine supernatants were not analyzed.

Isolation of Collagen from Osteoarthritic Cartilage

Type II collagen was isolated from osteoarthritic cartilage according to a method of Kuijer et al.²¹ Cartilage powder was extracted in 50 mM Tris-HCl, pH 7.4, containing 4 M guanidinium-HCl for 2×24 hours, followed by 30 minutes centrifugation at 60,000g. The pellet was washed three times in ice-cold distilled water and suspended in 0.5 M HAc containing 1 g pepsin per 20 g wet-weight cartilage (final pepsin concentration, 1 mg/mL). This was incubated for 24 hours at 4°C , followed by 30-minute centrifugation at 60,000g. The supernatant was

collected, and type II collagen was precipitated by dialyzing against 0.5 M HAc + 0.9 M NaCl for 24 hours at 4°C, followed by 30-minute centrifugation at 60,000g. The precipitate was dissolved in 0.5 M HAc, dialyzed against 0.1 M HAc, and lyophilized. The dried collagen was stored at -80°C until further use.

Western Blot Analysis

Analysis of collagen and the collagen fragments was performed by means of SDS-PAGE with the use of 10% and 20% polyacrylamide slab gels, followed by Western blot analysis. As a control, the pepsin solution used for the extraction of type II collagen was also analyzed by means of Western blot analysis. Unless otherwise mentioned, the protein concentration used was always 1 mg/mL. A blocking step was performed by incubation of the blotting membranes for 1 hour at room temperature in Tris-buffered saline (TBS; 20 mM Tris, 0.5 M NaCl, pH 7.5) containing 2% skimmed milk powder. This was followed by overnight incubation at room temperature in TBS + 0.05% Tween-20 (TBST), containing mouse-anti-type II collagen Ab-3, mouse-anti-type II collagen Ab-2, which binds on the C-terminal 1/4 part of type II collagen (Lab Vision), or rabbit-anti-col2-3/4C-short. Negative controls were incubated overnight at room temperature in TBST without the primary antibody. Secondary antibodies were goat-anti-mouse and mouse-anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., Carpinteria, CA), and tertiary antibodies were alkaline phosphatase (AP) conjugated rabbit-anti-goat (Jackson ImmunoResearch Laboratories, Inc.) and AP conjugated goat-anti-mouse (Bio-Rad, Hercules, CA). Secondary and tertiary antibodies were diluted 1:1000 in TBST, and the incubation time was 1 hour for each. Between the different antibody incubations, the membranes were washed 3 × 5 minutes in TBST. After the third antibody incubation step, the membranes were washed 2 × 5 minutes in TBST and 2 × 5 minutes in AP-buffer (20 mM Tris, 20 mM NaCl, 1 mM MgCl₂, pH 9.5). Protein bands were visualized by incubation in nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Bio-Rad).

Slot Blot Analysis

Slot blot analysis was performed by applying 20 µL of either pepsin-extracted vitreous collagen or unconcentrated vitreous supernatant into the slots of the slot-blot apparatus (Bio-Rad). As a positive control for the presence of col2-3/4C-short, cartilage type II collagen was cleaved with MMP-8, and 20 µL was then applied. The membrane was blocked by incubation for 1 hour in 5% skimmed milk and then was stained with the same antibodies and protocol used for Western blot staining.

Densitometry of Collagen Bands

Relative amounts of collagen in the pepsin extracts from the vitreous of the different age groups were determined by means of densitometry performed with a computer program (Quantiscan; Biosoft, Cambridge, UK) according to the manufacturer's instructions. A dilution series was made of P_{25-P}, of which the concentration of 1 mg/mL was arbitrarily set to 100%, and these were electrophoresed next to the samples. The final immunoblot was scanned, and the relative protein concentrations in P_{45-P} and P_{65-P} were calculated (Quantiscan; Biosoft).

Collagenase Treatment

To confirm the collagen nature of the isolated fragments, the samples were treated with collagenase type VII from *Clostridium histolyticum* (Sigma-Aldrich). Two 20-µL aliquots of protein sample from P_{25-P} were applied onto separate 10-kDa molecular weight cutoff filtration columns (Amicon Ultra; Millipore), and 500 µL TESCA buffer (50 mM TES [Sigma-Aldrich], 0.36 mM CaCl₂, pH 7.5) was added to each. This was filtrated by centrifugation at 4000g until a volume of approximately 100 µL was left on the filter, after which these washing steps were repeated. The pH was checked for neutrality by pH indicator paper. Ten units of collagenase in TESCA buffer were added to one sample,

and the same volume of TESCA buffer without the enzyme was added to the control sample, and both were incubated at 37°C for 5 hours. TESCA buffer was then replaced by 0.5 M HAc according to the same washing procedure used on the 10-kDa filtration column but with the addition of 0.5 M HAc. Both samples were lyophilized and redissolved in the original volume of 20 µL of 0.5 M HAc before Western blot analysis.

MMP Breakdown of Type II Collagen

To determine whether the breakdown products that naturally occur in the vitreous could result from MMP breakdown of type II collagen, we tested a series of MMPs that are known to be present in the vitreous, along with combinations thereof, for their ability to cleave type II collagen. Pepsin-extracted type II collagen from osteoarthritic cartilage was incubated with MMP-1, MMP-2, MMP-8, or MMP-9 alone or MMP-8 in combination with MMP-2, MMP-9, or both. MMPs were activated by mixing in 1 mM final concentration of 4-aminophenylmercuric acetate and by incubation for 1 or 2 hours at room temperature, depending on the manufacturer's instructions. Then, of each MMP, 0.5 U was added to 20 µg protein, and the mixtures were incubated for up to 2 hours at 37°C. All MMPs were obtained from R&D Systems (Minneapolis, MN).

Pepsin Breakdown of Denatured Type II Collagen

Pepsin, used in our collagen extraction procedure, is likely able to degrade denatured type II collagen and can, therefore, also affect the presence and sizes of naturally occurring breakdown products in the vitreous. To evaluate this possibility, we subjected degraded type II collagen to incubation with pepsin. First, pepsin-extracted type II collagen from osteoarthritic cartilage was incubated for 1 hour with MMP-8, as described. Then half the mixture was washed three times with 0.5 M HAc using a 50 kDa filter (Amicon Ultra; Millipore), which leaves intact type II collagen as well as col2-3/4C-short on top of the filter. Pepsin was added in a final concentration of 1 mg/mL in 0.5 M HAc to half of this sample. As a control, the other half of the sample consisted of 0.5 M HAc without pepsin. These samples were incubated for 24 hours at 4°C, similar to the pepsin extraction procedure applied to type II collagen from human vitreous. Both samples consisted of 20 µg type II collagen in a volume of 150 µL.

The other half of the MMP-8 cleaved mixture was used for analysis of the denaturation by incubation with α-chymotrypsin because this enzyme can fully degrade denatured type II collagen.²² First, EDTA was added in a final concentration of 2 mM to inactivate MMP-8. Then α-chymotrypsin was added to half this sample in a final concentration of 0.1 mg/mL in 50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, and 0.05% Brij-35 (TCNB). As a control, TCNB without the enzyme was added to the other half of the sample. The mixtures were incubated for 1 hour at 37°C. These samples also consisted of 20 µg type II collagen in a volume of 150 µL. All samples were subjected to Western blot analysis for the presence of type II collagen and col2-3/4C-short.

RESULTS

Immunohistochemistry

Immunohistochemistry repeatedly resulted in positive staining of all three vitreous bodies when the 3/4C-short antibody was used on sections of a human eye (Fig. 1A). The staining pattern was similar to, but weaker than, the type II collagen-stained sections of the same eye (Fig. 1C). Both type II collagen and col2-3/4C-short stained darkest in the vitreous cortex, where collagen concentrations are higher than in the central vitreous.¹ Figures 1A to 1D show the results from the 55-year-old donor. Healthy-appearing cartilage from an osteoarthritic knee, which was used as a negative control, showed no binding of the col2-3/4C-short antibody (Fig. 1F), whereas the positive-

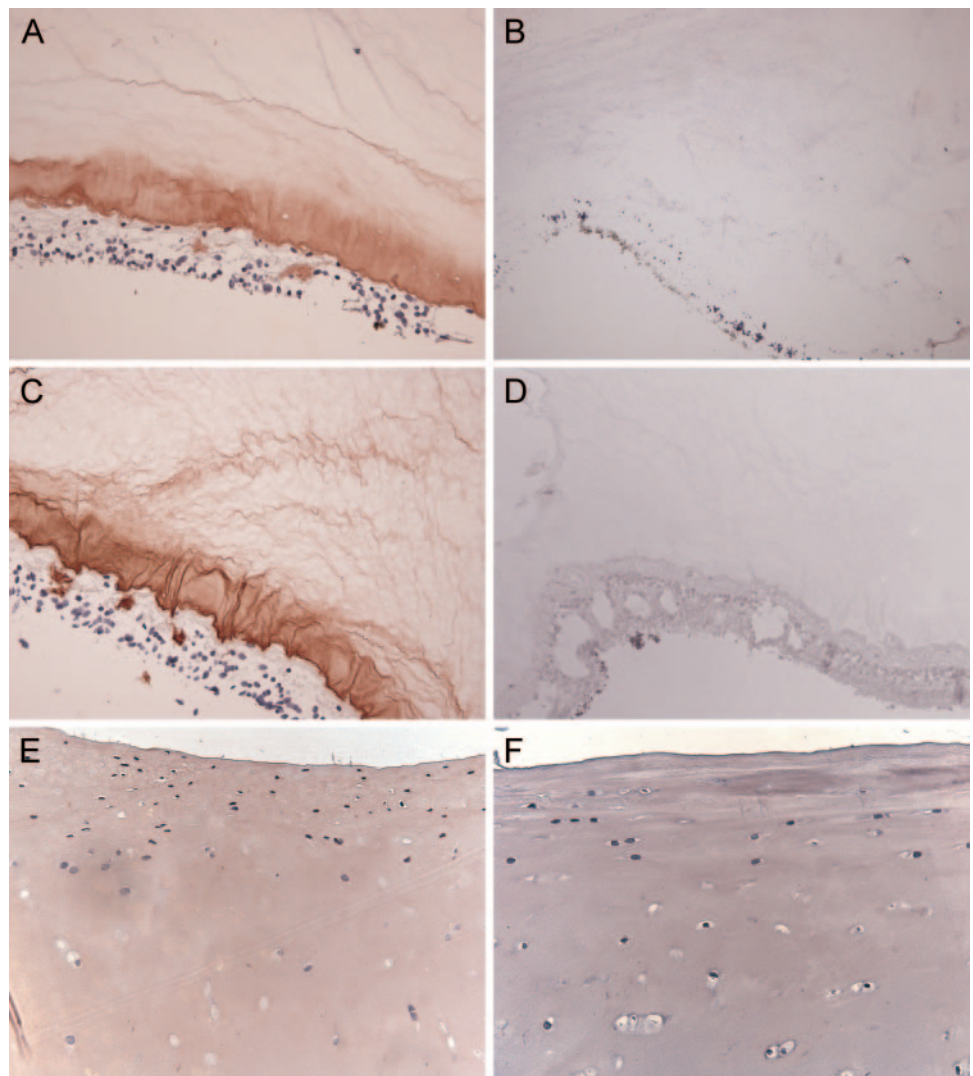


FIGURE 1. Immunohistochemistry of human vitreous and human cartilage. Eye sections were analyzed for the presence of (A) col2-3/4C-short and (C) type II collagen. The staining patterns of these antibodies were similar, though the intensity of the col2-3/4C-short staining was weaker. Negative controls of the eye sections (B, col2-3/4C-short; D, type II collagen) showed no aspecific binding of secondary antibodies. The specificity of the antibody against col2-3/4C-short was tested on (E) osteoarthritic and (F) healthy cartilage sections. Staining was faint in the osteoarthritic cartilage and absent from the healthy cartilage. Antibodies used were (C) mouse-anti-type II collagen (ab-3) and (A, E) rabbit-anti-col2-3/4C-short.

control osteoarthritic cartilage areas from the same knee did stain (Fig. 1E).

Western Blot Analysis

Protein extracts obtained from the pellets of centrifuged pools of vitreous bodies (P_{25-P} , P_{45-P} , and P_{65-P}) were subjected to Western blot analysis. By using the mouse antibody against type II collagen, intact type II collagen and several fragments of type II collagen were found in all age groups (Fig. 2). Although the protein concentrations used in all age groups were equal, both type II collagen and its breakdown products presented as more intensely stained bands in the younger pools. When P_{25-P} was set arbitrarily at 100%, the average type II collagen concentrations in P_{45-P} and P_{65-P} were 21% and 10%, respectively, as shown by densitometry. The supernatant that was obtained after centrifugation of the vitreous body pools was subjected to Western blot analysis using the same type II collagen antibody. No type II collagen fragments were detected in either the untreated supernatants or in the concentrated supernatants (data not shown). To confirm the collagenous nature of the fragments detected by Western blot analysis, an aliquot of P_{25-P} was treated with bacterial collagenase VII. Figure 3 shows that all fragments indeed disappear after collagenase digestion. Western blot analysis of the pepsin solution showed that this solution did not immunologically interfere with type II collagen and the range of breakdown products (not shown).

Western blot analysis of proteins isolated from the vitreous pellets and supernatants were also stained with the specific col2-3/4C-short antibody. In addition, concentrated supernatants were analyzed to detect the possible presence of the specific neopeptide on type II collagen fragments smaller than 50 kDa. Earlier attempts had demonstrated that the fraction containing proteins larger than 50 kDa could not be concentrated that much because of the high noncollagenous protein content, which led to nonspecific staining of the blotting membrane (not shown). In all cases, no specific binding was observed. The antibody did bind when a control sample consisting of vitreous type II collagen cleaved by MMP-8 was used, thereby confirming its ability to recognize the specific neopeptide by Western blot analysis (Fig. 4). A 20% gel was used to detect possible small fragments that contained the neopeptide, but no bands were found (not shown).

Slot Blot Analysis

We hypothesized that the fragments containing the col2-3/4C-short epitope could be undetectable by Western blot analysis because they were so small they would run off the SDS-PAGE gels or they were bound to or aggregated with other proteins. Therefore, we decided to perform slot blot analysis with the vitreous supernatants obtained after centrifugation and with the isolated vitreous collagen. Specific binding of the antibody was seen as a result (Fig. 5). Staining of the supernatants was

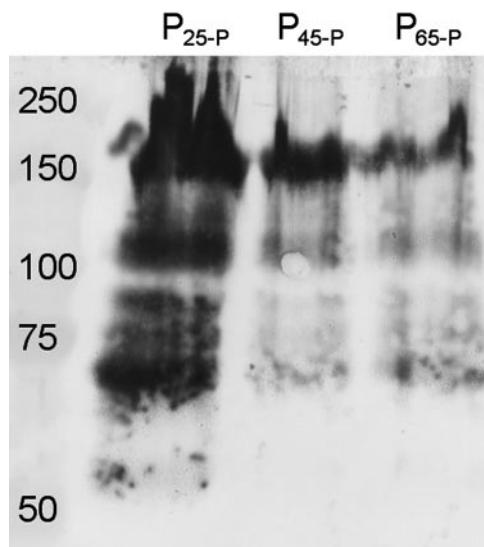


FIGURE 2. Western blot analysis shows an apparent decrease in type II collagen and its breakdown products in the vitreous with age. In human vitreous, type II collagen and its breakdown products are present in the pepsin-extracted pellets of all age groups. Compared with total concentrations of isolated protein, the darkest stained type II collagen bands were found in the younger age group. P_{25-P}, P_{45-P}, and P_{65-P} are the different pools, each consisting of four vitreous bodies. The values after P indicate the mean ages of the donors. Staining was performed with mouse-anti-type II collagen (ab-3). *Left lane*, molecular weight marker.

light, but specific, and hardly showed any variation with aging. The staining intensity of the pepsin extracts decreased with aging, finally resulting in an even lighter staining than found in the supernatant of P₆₅. The negative control of the slot blot was blank.

Type II Collagen Breakdown by Commercially Available MMPs

Because we were interested in the range and nature of type II collagen breakdown products found in the vitreous pools, we tried to simulate the breakdown process with commercially available MMPs known to occur in the vitreous body (MMP-1, MMP-2, MMP-8, MMP-9)^{18,19} (Fig. 6). Cleavage of type II collagen isolated from osteoarthritic cartilage by MMP-1 and MMP-8 resulted in the specific breakdown product col2-3/4C-short, but not in the naturally occurring breakdown products in the vitreous. An MMP-8 cleaved sample was used for further degradation by MMP-2 and MMP-9. This resulted in the complete disappearance of all type II collagen fragments. Incubation with MMP-2 alone resulted in complete degradation of type II collagen, thereby forming a weakly stained band between 50 and 75 kDa, but not the banding pattern seen in the vitreous. A reduction in staining of the intact type II collagen band was seen after 120 minutes of incubation with MMP-9 alone, but no clear new breakdown products were formed.

Comparison of Type II Collagen Breakdown Products in Vitreous and Cartilage

To investigate whether type II collagen degradation in the vitreous body occurred as in other type II collagen-containing tissues, we compared the vitreous breakdown products with breakdown products that naturally occur in osteoarthritic articular cartilage. These collagen fragments appeared to be of similar size (Fig. 7).

Effect of Postmortem Time

On Western blot analysis, porcine eyes showed a pattern of type II collagen breakdown products different from that of human eyes. Storage of porcine eyes at 4°C for 24 hours resulted in slightly more lightly stained bands of the breakdown products. One-week storage at 4°C resulted in a further reduction in staining. No new breakdown products were formed (Fig. 8).

Type II Collagen Breakdown by Pepsin

Incubation of MMP-8-cleaved type II collagen with pepsin resulted in the formation of two visible breakdown products (Fig. 9A), comparable in size to two of the protein bands found in pepsin-extracted vitreous type II collagen (86 kDa and 57 kDa; Fig. 9C). These newly formed bands did not bind to the col2-3/4C-short antibody (Fig. 9B). When MMP-8 cleaved type II collagen was incubated with α -chymotrypsin, all protein bands on Western blot analysis disappeared, thereby confirming that type II collagen had denatured after cleavage by MMP-8.

DISCUSSION

With the use of three different techniques, immunohistochemistry, Western blot analysis, and slot blot analysis, we found indications of active enzymatic breakdown of type II collagen in the human vitreous body. We also found evidence of MMP involvement, but MMPs alone could not account for all our observations.

Immunohistochemistry showed the presence of the specific type II collagen breakdown product col2-3/4C-short in the vitreous.

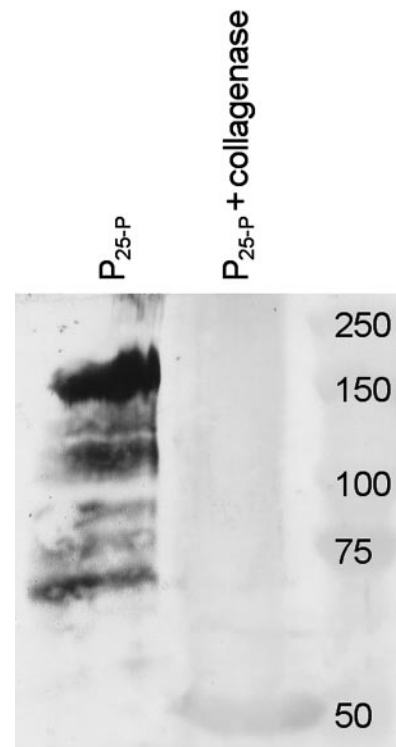


FIGURE 3. Western blot showing that collagenase treatment confirms the collagen nature of the vitreous fragments. Addition of collagenase type VII to the pepsin extract of the vitreous pool P₂₅ resulted in complete disappearance of all protein bands. This validated their collagenous origin. *Right lane*, molecular weight marker. The blot was stained with mouse-anti-type II collagen (ab-3).

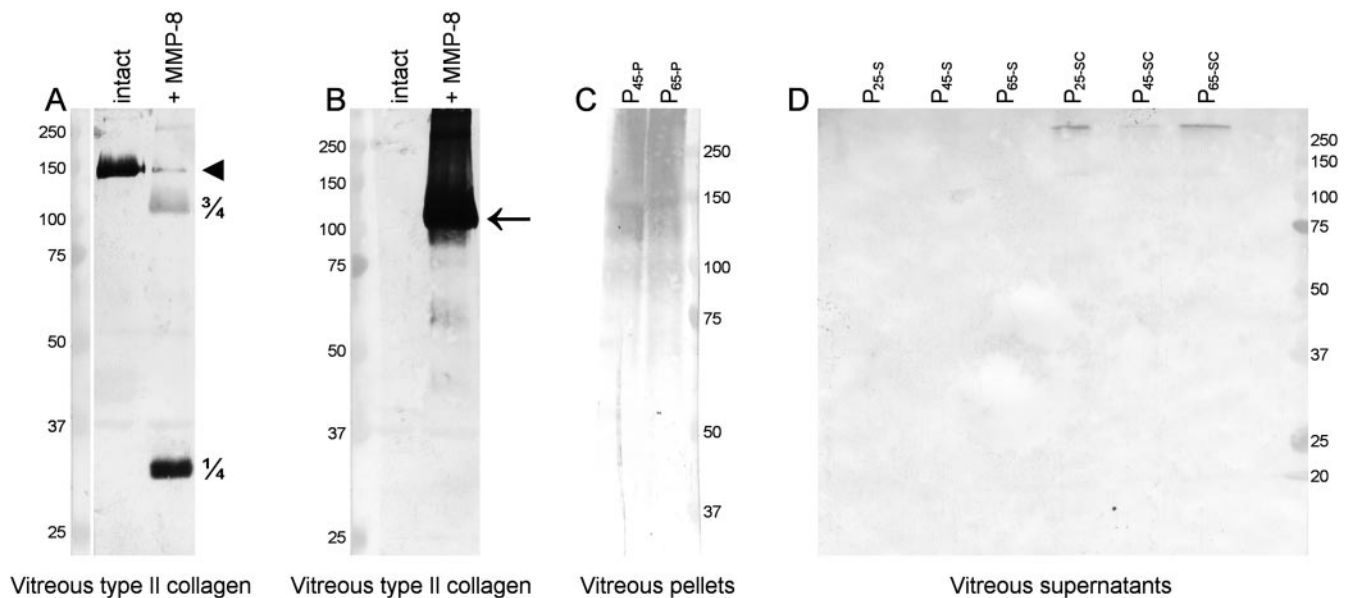


FIGURE 4. Western blot analysis of the vitreous for the presence of col2-3/4C-short. The specific antibody against col2-3/4C-short was tested for its functionality in Western blot analysis. (A) Therefore, intact type II collagen from the vitreous (*arrowhead*) was cleaved with MMP-8, which resulted in the disappearance of intact type II collagen and the appearance of the specific 3/4- and 1/4-breakdown products. Stained with mouse-anti-type II collagen (ab-2). Some aspecific staining was seen on the 3/4 breakdown product. (B–D) Stained with the rabbit antibody against col2-3/4C-short. (B) This antibody binds to the 3/4-breakdown product (*arrow*) but not the 1/4-breakdown product. (C, D) Binding of the col2-3/4C-short was not found in the pepsin extracts of the vitreous pellets or in the supernatants. Molecular weight markers are shown in either the *far right* or the *far left* lane of each Western blot.

ous, pointing to MMP-induced breakdown of type II collagen. The MMP types that cleave intact type II collagen between amino acids Gly775 and Leu/Ile776 are MMP-1, MMP-8, and MMP-13. Because MMP-1 and MMP-8 are found in the vitreous,^{18,19} it is likely that these are also active in the vitreous.

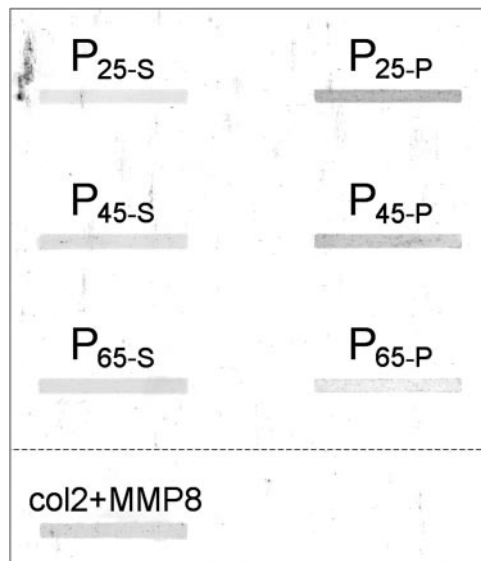


FIGURE 5. Slot blot of vitreous samples from pools P₂₅, P₄₅, and P₆₅. Supernatants (P_{25-S}, P_{45-S}, and P_{65-S}) and pepsin extracts (P_{25-P}, P_{45-P}, and P_{65-P}) were slot blotted, and the filter was stained with the rabbit-anti-col2-3/4C-short antibody. With aging, the fragment col2-3/4C-short decreased in the pepsin extracts until in P_{65-P} the detectable concentration was lower than found in the supernatants. No obvious differences in staining intensity can be seen in the supernatants. Adding MMP-8 to purified type II collagen from cartilage (col2+MMP8), which served as a positive control, gave specific staining of this band, thereby confirming the presence of col2-3/4C-short.

Western blot analysis demonstrated a range of type II collagen degradation products, but none of these contained the col2-3/4C-short epitope that we detected on blot after MMP-8 digestion of type II collagen. We ran our samples on 20% acrylamide gels. This limited the minimal size of proteins to be detected to approximately 10 kDa. It is possible that, after initial cleavage by MMP-1 or MMP-8, the resultant breakdown products were further cleaved by the same or other enzymes. In addition, pepsin extraction might have resulted in a loss of the col2-3/4C-short epitope. Figure 9 shows that the MMP-8-induced type II collagen breakdown product is further cleaved by pepsin and that the resultant smaller type II collagen breakdown products cannot be detected by the specific col2-3/4C-short antibody. Epitopes that bind antibodies can be as small as 6 amino acids; therefore, fragments recognized by antibodies can theoretically be of such low molecular weight that they are missed by Western blot analysis. Therefore, extensive enzymatic breakdown may result in positive immunohistochemistry but negative Western blot analysis. By slot blot analysis, which enables detection of smaller fragments, the specific neopeptide was detected, thereby confirming the immunohistochemical data. The neopeptide was detected primarily in the pepsin extracts. Furthermore, the pepsin extracts showed an age-related decrease of neopeptide so that, as a result, at an average age of 65 years, the concentration was even lower than in the supernatants. This indicated that at a young age type II collagen is already cleaved by MMPs and that the formed fragments are likely even further digested enzymatically. It was possible that the smallest neopeptide-containing fragments could be too small to stick to the blotting membrane and, therefore, could be undetectable. In future research, enzyme-linked immunosorbent assay should be used for the quantification of all neopeptide-containing fragments.

The breakdown products detected by Western blot analysis in the different age groups were similarly sized, but the amounts of the intact type II collagen and of its breakdown products seemed to decrease with aging, even when similar

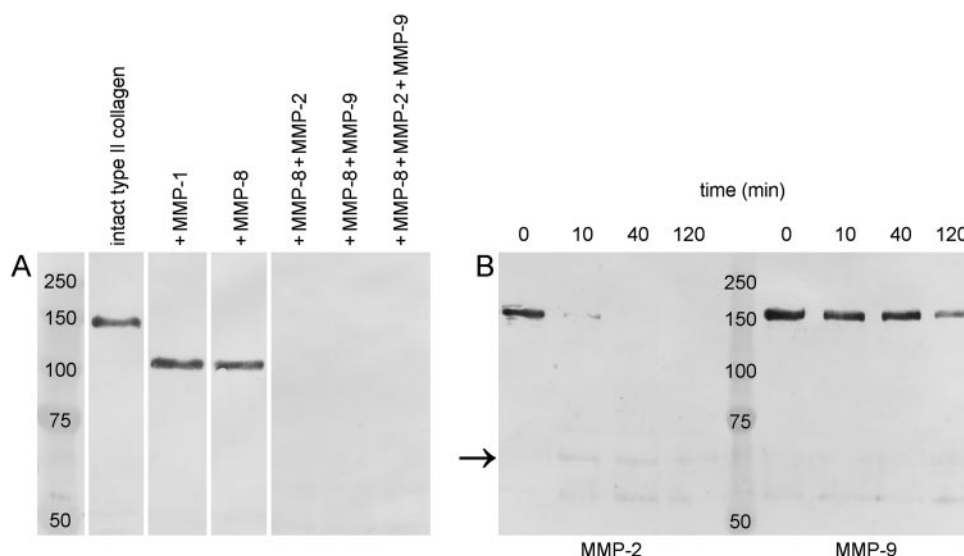


FIGURE 6. Western blot showing degradation by MMP-1, MMP-2, MMP-8, and MMP-9 of intact pepsin extracted type II collagen from osteoarthritic cartilage. (A) Cleavage by MMP-1 and MMP-8 resulted in the specific 3/4 breakdown product. When MMP-8 was combined with MMP-2, MMP-9, or both, this product also disappeared. (B) Incubation with MMP-2 resulted in the quick disappearance of intact type II collagen and the formation of a weakly stained product at approximately 65 kDa (*arrow*). Type II collagen appeared more resistant to MMP-9 but here also the amount of collagen decreased over time, though no clear new breakdown products were formed. The antibody used was mouse-anti-type II collagen (ab-3).

protein amounts were loaded on the gel. This seemed to contradict earlier studies describing a constant collagen content in the vitreous during aging.^{23,24} Three scenarios might have contributed to this observation. First, the accumulation of advanced glycation end products (AGEs), which we observed in the vitreous body,²⁵ can result in lower extractable collagen concentrations. In other ECMs that contain long-lived proteins, it has been demonstrated that AGEs negatively affect pepsin digestion of the matrix, thereby resulting in lower extractable amounts of collagen²⁶ and possibly of collagen fragments. Second, at least part of this decrease in protein concentrations can be explained by the rise in noncollagenous proteins in the vitreous that occurs with aging, resulting in a shift in the ratio

of collagenous and noncollagenous proteins. Previous research has shown that noncollagenous protein concentrations increase by approximately 50% from 10 to 50 years to 50 to 80 years.²³ In our own experiments, we found an increase in noncollagenous proteins of 50% from the age of approximately 20 years to approximately 60 years.²⁵ Third, some AGEs can absorb light of 280 nm,^{27,28} the wavelength we used to determine protein concentrations, and this might have caused inaccuracies in our measurements. The higher amount of AGEs seen in older vitreous bodies can therefore be mistaken for a higher protein concentration of the sample. However, because of the small amounts of protein that we could extract from the

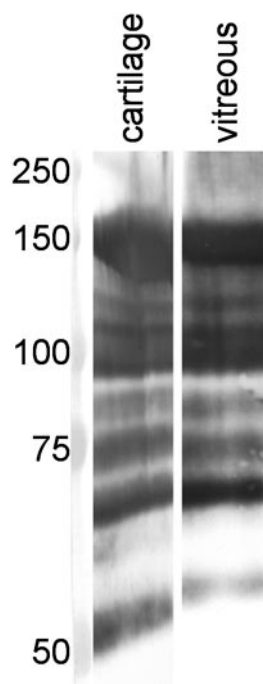


FIGURE 7. Vitreous and osteoarthritic cartilage contain similar type II collagen breakdown products. Comparison of type II collagen breakdown products from vitreous (P_{25-P}) and osteoarthritic cartilage by Western blot analysis. The blot was stained with mouse-anti-type II collagen (ab-3).

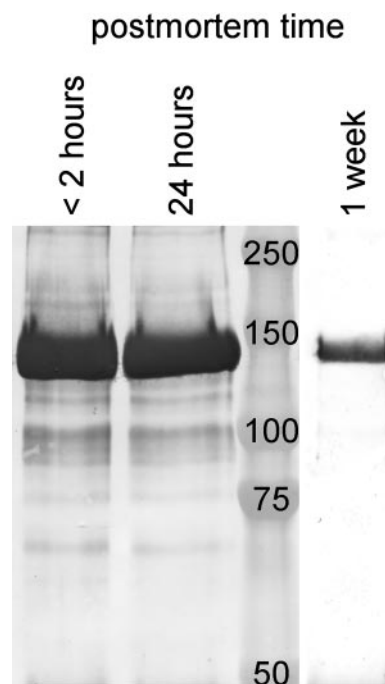


FIGURE 8. Western blot showing the effect of postmortem time on porcine vitreous. Storage of porcine eyes for 24 hours at 4°C resulted in a slight reduction in staining intensity of some type II collagen breakdown products. One-week storage at 4°C resulted in further reduction in staining intensity. No new breakdown products were found. The blot was stained with mouse-anti-type II collagen (ab-3).

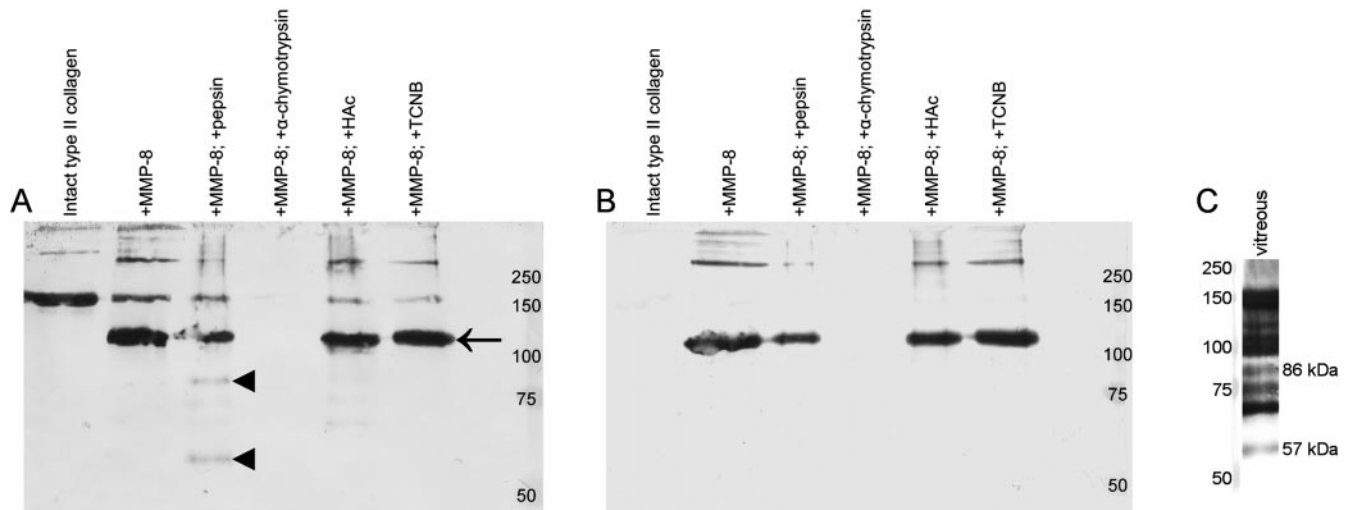


FIGURE 9. Western blot showing the effect of pepsin on denatured type II collagen. **(A)** Stained with mouse-anti-type II collagen (ab-3). **(B)** Stained with rabbit-anti-col2-3/4C-short. Incubation of intact type II collagen with MMP-8 resulted in the formation of the breakdown product col2-3/4C-short (arrow). **(A, arrowheads)** Subsequent incubation with pepsin resulted in some degradation of col2-3/4C-short, thereby forming two new visible breakdown products **(C)** comparable in size to the 86-kDa and 57-kDa bands of the vitreous. **(B)** Staining of these breakdown products was absent. Incubation of the MMP-8-degraded type II collagen with α -chymotrypsin resulted in the complete disappearance of all protein bands, thereby confirming that pretreatment with MMP-8 resulted in the denaturation of type II collagen. Control samples, which were incubated with HAc and TCNB instead of pepsin and α -chymotrypsin, respectively, showed that the procedures following incubation with MMP-8 did not affect the collagen concentration in the samples.

human vitreous, we were obliged to use this method to estimate protein concentrations.

The possible effects of aging changes on the extractability of collagen breakdown products makes it difficult to establish a direct relationship between the amount of synchysis and the extent of enzymatic breakdown in the vitreous. In addition, it is likely that the breakdown products, as shown by Western blot analysis, are intermediate products that will be cleaved further. Whether early syneresis in highly myopic eyes is caused by more extensive enzymatic collagen breakdown cannot be deduced from this study because only eyes of apparently normal size were included.

One question that remains unresolved is which enzyme or enzymes are responsible for the type II collagen breakdown products we found by Western blot analysis. Cleavage of type II collagen by MMP-1 and MMP-8, both known to be present in the vitreous, did not produce these type II collagen breakdown products, and a combination of MMP-8 with MMP-2 or MMP-9, or both, produced such extensive breakdown that clear fragments were no longer detectable by Western blot analysis. The weakly stained band that was formed on degradation by MMP-2 alone could correspond to one of the naturally occurring breakdown products in the vitreous, but MMP-2 cannot explain the range of other breakdown products that we repeatedly found. Although MMP-9 was able to digest some of the type II collagen, we did not find any larger fragments. MMP-9, though, is not supposed to be able to cleave the collagen triple helix.²⁹ It is possible that our type II collagen, which was isolated from diseased osteoarthritic cartilage, contained some monomeric collagen that could be degraded by MMP-9. Together, these observations suggest that other type II collagen degrading enzymes are present in the vitreous. However, we cannot exclude that in the combined MMP experiments, the concentrations of MMP-2 and MMP-9 were so high that possible intermediate breakdown products were degraded too quickly for detection. Such breakdown products might well be detectable in the presence of lower MMP concentrations. Furthermore, an effect of pepsin extraction on the sizes of the breakdown products cannot be fully excluded. We showed

that digestion by pepsin of MMP-8-cleaved type II collagen resulted in the formation of two breakdown products sized similarly to those found in pepsin-extracted vitreous type II collagen. The other breakdown products found in the vitreous extract could not be reproduced by pepsin digestion. Given that collagen can become denatured in other ways—enzymatically,³⁰ by free radicals,³¹ or by physical denaturation³²—either before or during the extraction procedure, we cannot completely exclude any effects of these on the observed protein bands. Future studies are needed to examine these possibilities.

In the study with porcine eyes we found that storage of intact eyes for up to 1 week after death at 4°C did not result in the formation of additional type II collagen breakdown products in the vitreous. Porcine eyes were chosen for this study because ages and postmortem times could be standardized. However, we realize that porcine and human eyes are not equal and can, for example, show differences in types and expression of proteinases. Therefore, postmortem effects in our results using human eyes cannot fully be excluded.

In conclusion, our results provide evidence that enzymatic breakdown of the collagen fibrils is probably one of the mechanisms that causes age-related changes in the vitreous. Enzymatic activity in the vitreous was already hypothesized by Bishop et al.,³ who found a loss of proteoglycans from the collagen fibrils with aging caused by enzymatic breakdown. Proteoglycan loss and type II collagen breakdown are also seen in osteoarthritic cartilage, where collagen breakdown products are found primarily at sites where proteoglycans are depleted.^{33,34} This, combined with the finding that type II collagen breakdown products from the vitreous are similar to those in cartilage, suggests that similar enzymes may be involved in vitreous and cartilage collagen breakdown. Future research should be directed at identifying these enzymes.

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